CLAIMS

1. A method for constructing a variant of a parent subtilase, wherein the variant has at least one altered property as compared to said parent subtilase, which method comprises:

a) analyzing the three-dimensional structure of the parent subtilase to identify, on the basis of an evaluation of structural considerations in relation to a JP170 three dimensional structure, at least one amino acid residue or at least one structural region of the subtilase, which is of relevance for altering said property;

b) modifying the DNA of the polynucleotide encoding the parent to construct a polynucleotide encoding a variant subtilase, which in comparison to the parent subtilase, has been modified by deletion, substitution or insertion of the amino acid residue or structural part identified in i) so as to alter said property;

c) expressing the variant subtilase in a suitable host, and

d) testing the resulting subtilase variant for said property.

2. A method of producing a subtilase variant, wherein the variant has at least one altered property as compared to a parent subtilase, which method comprises:

a) producing a model structure of the parent subtilase on the three-dimensional structure of BPN', TY145 or JP170; or producing an actually determined three-dimensional structure of the parent subtilase,

b) comparing the model or actual three-dimensional structure of the parent subtilase to the JP170 structure by superimposing the structures through matching the CA, CB, C, O, and N atoms of the active site residues,

c) identifying on the basis of the comparison in step b) at least one structural part of the parent subtilase, wherein an alteration in said structural part is predicted to result in an altered property;

d) modifying the nucleic acid sequence encoding the parent subtilase to produce a nucleic acid sequence encoding at least one deletion or substitution of one or more amino acids at a position corresponding to said structural part, or at least one insertion of one or more amino acid residues in positions corresponding to said structural part;

e) performing steps c) and d) iteratively N times, where N is an integer with the value of one or more;

f) preparing the variant resulting from steps a) - e);

g) testing the properties of said variant; and

h) optionally repeating steps a) - g) recursively; and

i) selecting a subtilase variant having at least one altered property as compared to the parent subtilase.

j) expressing the modified nucleic acid sequence in a host cell to produce the variant subtilase;

k) isolating the produced subtilase variant;

l) purifying the isolated subtilase variant and

m) recovering the purified subtilase variant.

3. The method of claims 1 or 2, wherein the parent subtilase belongs to the sub-group I-S1, preferably selected from the group consisting of ABSS168, BASBPN, BSSDY, and BLSCAR, or functional variants thereof having retained the characteristic of sub-group I-S1.

4. The method of claims 1 or 2, wherein the parent subtilase belongs to the sub-group I-S2, preferably selected from the group consisting of BLS147, BLS309, BAPB92, and BYSYAB, or functional variants thereof having retained the characteristic of sub-group I-S2.

5. The method of claims 1 or 2, wherein the parent subtilase belongs to the TY145 type subgroup, preferably selected from the group comprising TY145, S39 and S41.

6. The method of claims 1 or 2, wherein the parent subtilase belongs to the JP170 type subgroup, preferably selected from the group comprising JP170, proteases KP43, KP1790, KP9860, Protease Ya, Protease E-1 and SD-521

7. The method of claim 6, wherein the parent JP170 type subtilase that is modelled is at least 58% homologous to SEQ ID NO:1, preferably at least 60% homologous, more preferably at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, more preferably at least 93%, more preferably at least 94%, more preferably at least 95%, more preferably at least 96%, more preferably at least 97%, more preferably at least 98% or even more preferably at least 99% homologous to the sequence of SEQ ID NO:1.

8. The method according to claims 6 or 7, wherein the parent JP170 subtilase is a JP170 like subtilase which is at least 58% homologous to the sequence of SEQ ID NO:1, comprising the overall subtilisin fold and the following structural characteristics:

a) a twisted beta-sheet with 7 strands,

b) six alpha helices,

c) at least three ion-binding sites,

and not comprising the Strong and Weak ion-binding sites of the BPN' like subtilases, wherein the positions of said three ion-binding sites in the three-dimensional structure of the subtilase is defined by the distance to the c-alpha atoms of the three active site amino acid residues of the subtilases, that is Ser, His and Asp, and the c-alpha atom of the amino acid residue next to the active site Ser residue (next to Ser), wherein said distances between:

I) ion-binding site 1 and

i) Asp c-alpha atom is 26.70-28.70Å,

ii) His c-alpha atom is 22.10-24.10Å,

iii) Ser c-alpha atom is 16.95-18.95Å,

iv) next to Ser c-alpha atom is 15.30-17.30Å,

II) ion-binding site 2 and

i) Asp c-alpha atom is 33.50-35.50Å,

ii) His c-alpha atom is 37-39Å,

iii) Ser c-alpha atom is 29.40-31.40Å,

iv) next to Ser c-alpha atom is 30.70-32.70Å,

III) ion-binding site 3 and

i) Asp c-alpha atom is 41.50-43.50Å,

ii) His c-alpha atom is 42.90-44.90Å,

iii) Ser c-alpha atom is 34.50-36.50Å,

iv) next to Ser c-alpha atom is 35-37Å.

9. The method of claim 1 or 2 for producing a JP170 type subtilase variant, wherein the variant has at least one altered property as compared to a parent subtilase, which method comprises:

n) producing a model structure of the parent JP170 type subtilase on the three-dimensional structure of JP170; or producing an actually determined three-dimensional structure of the parent subtilase,

o) comparing the model or actual three-dimensional structure of the parent JP170 type subtilase to the BPN' or TY145 structure by superimposing the structures through matching the CA, CB, C, O, and N atoms of the active site residues,

p) identifying on the basis of the comparison in step b) at least one structural part of the parent JP170 type subtilase, wherein an alteration in said structural part is predicted to result in an altered property;

q) modifying the nucleic acid sequence encoding the parent JP170 type subtilase to produce a nucleic acid sequence encoding at least one deletion or substitution of one or more amino acids at a posi- tion corresponding to said structural

part, or at least one insertion of one or more amino acid residues in positions corresponding to said structural part;

r) performing steps c) and d) iteratively N times, where N is an integer with the value of one or more;

s) preparing the JP170 type subtilase variant resulting from steps a) - e);

t) testing the properties of said variant; and

u) optionally repeating steps a) - g) recursively; and

v) selecting a JP170 type subtilase variant having at least one altered property as compared to the parent subtilase.

w) expressing the modified nucleic acid sequence in a host cell to produce the variant subtilase;

x) isolating the produced JP170 type subtilase variant;

y) purifying the isolated subtilase variant and

z) recovering the purified subtilase variant.

10. The method of claim 9, wherein step (c) identifies amino acid residue positions located at a distance of 10Å or less to the ion-binding site of the JP170 type parent, preferably positions located at a distance of 6 Å or less.

11. The method of claim 9, wherein step (c) identifies amino acid residue positions in the JP170 type parent, the modification of which provides for the removal of the ion binding site by modification of at least one of the positions identified.

12. The method of claim 9, wherein step (c) identifies amino acid residue positions in highly mobile regions of the JP170 type parent.

13. The method of claim 9, wherein step (c) identifies amino acid residue positions in mobile regions of the JP170 type parent.

14. The method of claim 9, wherein step (c) identifies amino acid residue positions in the parent JP170 type, the modification of which may create at least one disulfide bridge by insertion of or substitution with at lease one Cys residue.

15. The method of claim 9, wherein steps (c) and (d) provide for constructing a variant of a parent JP170 type having a modified surface charge distribution by:

c') identifying, on the surface of the parent JP170 type, at least one charged amino acid residue;

d') modifying the charged residue identified in step (a) through deletion or substitution with an uncharged amino acid residue;

16. The method of claim 9, wherein steps (c) and (d) provide for constructing a variant of a parent JP170 type having a modified surface charge distribution by:

c") identifying, on the surface of the parent JP170 type, at least one position being occupied by an uncharged amino acid residue;

d") modifying the charge in that position by substituting the uncharged amino acid residue with a charged amino acid residue or by insertion of a charged amino acid residue at the position.

17. The method of claim 9, wherein steps (c) and (d) provide for constructing a variant of a parent JP170 type having a modified surface charge distribution by:

c"') identifying, on the surface of the parent JP170 type, at least one charged amino acid residue;

d"') substituting the charged amino acid residue identified in step (a) with an amino acid residue having an opposite charge.

18. The method of claim 9, wherein step (c) identifies amino acid residue positions in the parent JP170 type, the modification of which to Pro may create a JP170 type variant exhibiting improved stability.

19. The method of claim 9, wherein step (c) identifies amino acid residue positions in the parent JP170 type at a distance of less than 10Å from the active site residues.

20. The method of one or more of claims 9 to 19, wherein N in step (e) is an integer between 1 and 50, 45, 40, 35, 30, 25, 20, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, or 2.

21. A JP170 type subtilase variant comprising at least one modification in an amino acid residue in a position located at a distance of 10Å or less to the ion-binding site, preferably positions located at a distance of 6 Å or less.

22. The variant of claim 21, wherein modifications are made in at least one of the positions::

a) ion-binding site 1: 183, 184, 185, 186, 187, 188, 189, 191, 193, 195, 196, 197, 198, 199, 200, 201, 202, 203, 224 and 225,

b) ion-binding site 2: 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392 and 393,

c) ion-binding site 3: 348, 350, 352, 363, 364, 365, 366, 367, 369, 370, 380, 381, 382, 383, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 414, 415, 416, 417, 418, 419, 420, preferably the modifications: S193Q,Y; H200D,N; H200D,N+D196N; N390D; N391D; G394N,Q,F,Y,S and W392S,N,Q.

23. A JP170 type subtilase variant comprising the introduction of a ion-binding site corresponding to the Strong ion-binding site of the BPN' like family subtilases, wherein said variant has a partial or full deletion of the region N79-N82 of SEQ ID NO:1 and subsequent insertion of one or more amino acid residues, preferably insertion of the sequence LNNSIQV (SEQ ID NO:5) followed by the substitution A45D,N and optionally the substitutions E44P,T and/or R47Q.

24. A JP170 type subtilase variant in which one or more ion-binding sites have been removed, wherein said variant comprises partial or full deletion of the region N186-N199 of SEQ ID NO:1 and subsequent insertion of one or more amino acid residues, preferably insertion of the sequence SSN (SEQ ID NO:6), and preferably further comprising one or both of the substitutions I7Q and V3Y.

25. A BPN' type subtilase variant in which the ion-binding sites has been removed, wherein said variant comprises:

a) partial or full deletion of the region A194-L196 (Savinase in BPN' numbering) or a corresponding region in another BPN' like subtilase and insertion of three or more amino acid residues, preferably insertion of P209-P217 from JP170 or a corresponding region in another JP170 like subtilase and

partial or full deletion of the region L75-L82 (Savinase in BPN' numbering) or a corresponding region in said other BPN' like subtilase and insertion of one or more amino acid residues, preferably insertion of H83-Y92 from TY145 or a corresponding region in another TY145 like subtilase or

b) partial or full deletion of the region A194-L196 (Savinase in BPN' numbering) or a corresponding region in another BPN' like subtilase and insertion of three or more amino acid residues, preferably insertion of P209-P217 from JP170 or a corresponding region in another JP170 like subtilase and

partial or full deletion of the L75-L82 (Savinase in BPN' numbering) or a corresponding region in said other BPN' like subtilase and insertion of one or more amino acid residues, preferably insertion of N79-K83 from JP170 or a corresponding region in another JP170 like subtilase.

26. A JP170 type subtilase variant comprising at least one modification in a

position selected from the group comprising positions:
13, 14, 15, 16, 17, 18, 37, 38, 39, 40, 41, 42, 43, 47, 48, 49, 50, 58, 59, 60, 67, 96, 97, 98, 99, 107, 108, 109, 110, 111, 131, 132, 133, 134, 152, 153, 163, 164, 165, 166, 188, 189, 190, 191, 193, 195, 210, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 326, 327, 328, 329, 330, 331, 332, 337, 338, 339, 340, 342, 355, 356, 357, 359, 360, 372, 373, 374, 375, 376, 377, 378, 384, 385, 387, 388, 389, 390, 391, 392, 404, 405, 406, 407, 408, 409, 410, 411 and 419.

27. The subtilase variant according to claim 26 comprising one or more of the modifications: W240H,Y; G355A,S; S356T,N; T357N,Q,D,E,P; A359S,T,N,Q or S360T,N.

28. A variant subtilase comprising an alteration in one or more positions which are within a distance of 10Å from a CI2 inhibitor which is bound to the active site of JP170, wherein the positions, as specified in SEQ ID NO:1 are:
29, 30, 31, 32, 64, 67, 68, 69, 70, 71, 72, 93, 96, 97, 98, 107, 108, 109, 110, 113, 114, 127, 128, 129, 130, 131, 132, 133, 134, 136, 138, 139, 140, 141, 144, 157, 174, 180, 181, 182, 183, 191, 193, 202, 203, 205, 206, 207, 211, 223, 224, 225, 226, 234, 235, 236, 237, 238, 239, 240, 241, 249, 250, 251, 252, 253, 254, 257, 258, preferably comprising the substitution W129L.

29. A JP170 like subtilase variant comprising one or more disulfide bridges introduced by one or more of the following modifications: G21C+A86C, V26C+A265C, G57C+G105C, G74C+A229C, Q111C+N143C, G160C+S170C, A286C+V349C, A27C+A122C, A45C+G78C, V72C+P258C, G78C+A229C, D98C+G104C, Q111C+Y147C, G135C+G167C, R142C+P354C, V144C+A178C, G182C+P217C, A183C+G223C, A195C+Y225C, F271C+P279C, A287C+A430C, A293C+T310C, E322C+S428C, S324C+A332C, S327C+P424C, D352C+N397C, G355C+T362C, G291C+S314C, Preferably one or more of the substitutions: G21C+A86C, V26C+A265C, G57C+G105C, G74C+A229C, Q111C+Y143C, G160C+S170C, A286C+V349C, A4C+P222C and A27C+A117C wherein the positions correspond to the positions in SEQ ID NO:1.

30. A JP170 type subtilase variant comprising an alteration in one or more of the positions N79, N316, L381, K9, and K313, preferably comprising one or more of the substitutions N79D, N316D, L381D, K9R, and K313R of SEQ ID NO:1.

31. A JP170 type subtilase variant comprising an alteration in one or more of the positions 22, 44, 110, 139, 140, 166, 198, 201, 203, 231, 282, 356, 357 and 378, preferably comprising one or more of the substitutions: $_{64}$Q22P, E44P, L110P, T139P, D140P,

S166P I198P, V201P, Q203P, S231P, S282P, S356P, T357P and K378P.

32. A JP170 like subtilase variant comprising a deletion of the region 311-433, preferably deletion of positions 317-433 or 315-433, further comprising one or more of the substitutions L283N,Q; A290S,N and W306H,Y,K.

33. An isolated nucleic acid sequence comprising a nucleic acid sequence, which encodes for the subtilase variant defined or produced in any of claims 21 to 32.

34. An isolated nucleic acid sequence according to claim 33, wherein the nucleic acid sequence is selected form the group consisting of:

a) a nucleic acid sequence encoding an enzyme having at least 58% homology with the amino acid sequence shown in SEQ ID NO:1, and

b) a nucleic acid sequence which hybridizes under low stringency conditions, preferably under medium stringency conditions, in particular under high stringency conditions, with a complementary strand of the nucleic acid sequence encoding an enzyme having at least 58% homology with the amino acid sequence shown in SEQ ID NO:1, or

c) a subsequence of any of a) or b) of at least 100 nucleotides.

35. An isolated nucleic acid construct comprising a nucleic acid sequence as defined in any of claims 33 or 34, operably linked to one or more control sequences capable of directing the expression of the polypeptide in a suitable expression host.

36. A recombinant host cell comprising the nucleic acid construct of claim 35.

37. A method for producing the variant defined in any of claims 21-32, the method comprising:

a) cultivating the recombinant host cell of claim 36 under conditions conducive to the production of the subtilase variant; and

b) recovering the variant.

38. A detergent composition comprising a JP170 type subtilase variant or a BPN' type subtilase variant of any of claims 21-32.

39. Use of a JP170 type subtilase variant or a BPN' type subtilase variant of claims 21-32 in cleaning or washing applications.